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Novel Defective Interfering DNAs Associated with Ageratum Yellow Vein Geminivirus Infection of *Ageratum conyzoides*[1]

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Defective DNA forms of the geminivirus ageratum yellow vein virus (AYVV) have been identified in naturally infected *Ageratum conyzoides* plants. Several examples of the defective DNA have been cloned from purified virus-specific supercoiled DNA and characterized by sequence analysis. All are approximately half the size of AYVV genomic DNA, and all contain intergenic region sequences and the 5′ terminus of gene *C1* as well as additional sequences that are unrelated to the viral genomic DNA. The chimeric nature of the defective DNA distinguishes it from previously characterized geminivirus defective and satellite DNAs. The defective DNA ameliorates disease symptoms and causes a significant delay in the accumulation of viral DNA during the early stage of infection when coinoculated with the AYVV genomic DNA into *Nicotiana benthamiana,* suggesting a biological role as a defective interfering DNA. © 1997 Academic Press

INTRODUCTION

Members of the *Geminiviridae* are unique plant viruses that have small circular single-stranded (ss) DNA genomes encapsidated in twinned (geminate) particles. They have been divided into three subgroups on the basis of genome organization, host range, and insect vector (Briddon and Markham, 1995), of which subgroup III contains the greatest number of distinct viruses. The first molecular analyses of subgroup III members, on African cassava mosaic virus (ACMV, formerly called cassava latent virus (CLV)) (Stanley, 1983; Stanley and Gay, 1983) and tomato golden mosaic virus (TGMV) (Hamilton *et al.* 1984), revealed two genomic components, designated DNA A and DNA B, both of which are essential for systemic infectivity. DNA A encodes the capsid protein and proteins required for viral DNA replication and the control of viral gene expression, while DNA B encodes proteins required for nuclear trafficking and cell-to-cell movement of the viral DNA (reviewed by Bisaro (1996) and Sanderfoot and Lazarowitz (1996)). Subsequent analyses demonstrated that the majority of subgroup III members have similar genome arrangements with minor differences correlating with geographic origin, either Old World or New World (Padidam *et al.,* 1995). More recently, however, a number of related viruses lacking a DNA B component, such as tomato yellow leaf curl virus (TYLCV) and tomato leaf curl virus (TLCV), have been isolated from tomato (Kheyr-Pour *et al.,* 1991; Navot *et al.,* 1991; Dry *et al.,* 1993). In these cases, either DNA A gene products perform the functions normally associated with DNA B or the viruses have evolved alternative mechanisms for movement within plants. The evolutionary relationship between viruses with monopartite and bipartite genomes is unclear, but one possibility is that DNA B has been lost when indigenous bipartite viruses became adapted to permissive hosts such as tomato. If this is the case, it is reasonable to assume that progenitors of such viruses may still exist in the indigenous hosts that serve as reservoirs for the virus.

For this reason we initiated an investigation on ageratum yellow vein virus (AYVV), a geminivirus that infects *Ageratum conyzoides* L., a common weed which thrives in moist, shady areas in many parts of south east Asia. AYVV infection is not detrimental to *A. conyzoides* growth and development, suggesting that the weed may act as a reservoir host for the maintenance of the virus population. To investigate the etiology of *A. conyzoides* yellow vein disease, we recently cloned and sequenced the genomic DNA of an AYVV isolate from Singapore (Tan *et al.,* 1995). Our results confirmed earlier indications based on insect transmission, host range, serology, and cytopathology (Tan and Wong, 1993; Wong *et al.,* 1993) that AYVV is indeed a subgroup III geminivirus, and is most closely related to TLCV (Dry *et al.,* 1993). The single genomic component was shown to produce symptoms typical of AYVV infection when agroinoculated into *Nicotiana benthamiana,* French bean, and tomato (Tan *et al.,* 1995). However, repeated attempts to reestablish an in-

[1] The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under Accession Nos. Y14167 and Y14168.

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fection in *A. conyzoides* by either agroinoculation or whitefly transmission were unsuccessful (Tan *et al.,* 1995). Our inability to formally demonstrate that AYVV is the causal agent of the disease in *A. conyzoides* led us to speculate that an additional factor, possibly an undetected second genomic component analogous to DNA B, is necessary for infection of this host.

Here, we have attempted to address this problem by purifying virus-specific supercoiled (sc) DNA with which to search for additional genomic components. We demonstrate that the scDNA does not contain detectable levels of a DNA B component. However, we have identified and characterized novel defective DNAs associated with infected *A. conyzoides.* We demonstrate that the defective DNA has a significant effect on AYVV DNA accumulation and symptom development when reintroduced into *N. benthamiana* by agroinoculation.

METHODS

Source and maintenance of virus isolate

A. conyzoides plants showing typical yellow vein symptoms were collected from wastelands in Singapore and the virus was maintained in *A. conyzoides* by *Bemisia tabaci* (Gennadius) transmission in insect-proof cages. AYVV was held and manipulated at the John Innes Centre under MAFF license numbers PHF 1419C/1907(6/96) and PHF 1419C/1922(4/96) under the Plant Pests (Great Britain) Order 1980.

Isolation of virus-specific scDNA

Total cellular nucleic acids were extracted from 50 g AYVV-infected *A. conyzoides* using the procedure of Covey and Hull (1981). ScDNA was purified by CsCl gradient centrifugation as described by Stanley and Townsend (1985). Gradient fractions containing scDNA were identified by Southern blot analysis of samples following fractionation in agarose gels containing TNE buffer (40 mM Tris–acetate, pH 7.5, 20 mM sodium acetate, 2 mM EDTA). Blots were hybridized with a probe corresponding to the full-length genomic DNA insert of pHN419 (Tan *et al.,* 1995) randomly labeled according to the procedure of Feinberg and Vogelstein (1983). Pooled fractions were dialyzed extensively against redistilled water at 4°C and the scDNA was precipitated with ethanol. The resulting dark brown precipitate was redissolved in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA), and the scDNA was further purified by tip-100 column chromatography (Qiagen) and precipitated according to the manufacturer's protocol. The resulting white precipitate was redissolved in 300 μl TE buffer.

Construction and characterization of cloned copies of AYVV defective DNA

The scDNA was digested with *Eco*RI and cloned into pIC19H (Marsh *et al.,* 1984). Clones containing viral DNA inserts were identified by colony hybridization using a probe specific for the AYVV intergenic region produced by PCR-amplification using primers CER001 and CER002 (refer to Fig. 3). Nucleotide sequences of cloned DNAs were derived by the dideoxynucleotide chain termination method of Sanger *et al.* (1977), using a T7 sequencing kit (Pharmacia) and [α-^{33}P]dATP (DuPont NEN). To avoid sequencing ambiguities, sequences were determined for both DNA strands. Sequences were assembled and analyzed using University of Wisconsin Genetics Computer Group software (Devereux *et al.,* 1984).

Agroinoculation of AYVV genomic and defective DNAs

The construction of clone pHNBin419 containing a partial repeat of AYVV genomic DNA in pBin19 (Bevan, 1984) has been described by Tan *et al.* (1995). To produce a partial repeat of defective DNA, clone pAYVdef17 (described under Results) was selected and digested with *Eco*RV. This served to remove sequences from the *Eco*RV sites to the *Eco*RI site within the clone insert (refer to Fig. 3) and additional vector sequences downstream as far as the polylinker *Eco*RV site, to produce pAYVdef17/0.2. The full-length defective DNA insert of pAYVdef17 was cloned into the now unique *Eco*RI site of the deletion mutant to produce pAYVdef17/1.2.

Clone pAYVdef17/1.2 contains two *Acl*I sites within the ampicillin resistance gene of the pIC19H vector and a single site within the defective DNA insert located immediately upstream of the *C1* initiation codon (refer to Fig. 3). The clone was linearized by partial *Acl*I digestion, and linear molecules were purified by agarose gel electrophoresis. Overlapping 5′ termini were blunt-ended using Klenow fragment and the plasmid was recircularized to insert two additional nucleotides and create an *Mlu*I site. Mutant pAYVdef17ΔC1/1.2, containing a frameshift mutation within the *C1* coding sequence, was selected with ampicillin following transformation of *Escherichia coli* strain JM83. The presence of the mutation was identified by *Mlu*I digestion and confirmed by sequence analysis.

The pAYVdef17/1.2 and pAYVdef17ΔC1/1.2 inserts were subcloned as *Hin*dIII fragments into pBin19 to produce pBinAYVdef17 and pBinAYVdef17ΔC1, respectively. Clones were conjugated into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.,* 1983) using pRK2013 as a helper plasmid (Ditta *et al.,* 1980).

AYVV genomic and defective DNA clones were introduced into *N. benthamiana* by agroinoculation as described by Tan *et al.* (1995). Plants were maintained in the UK in accordance with the requirements of the Advisory Committee on Genetic Manipulation, in an insect-free glasshouse at 25°C with supplementary lighting to give a 16-h photoperiod.

Inoculation of plants by particle bombardment

The construction of clone pHNIC419 containing a partial repeat of AYVV genomic DNA in pIC19H (Marsh *et al.,* 1984) has been described by Tan *et al.* (1995). Gold particles were coated with either purified scDNA or cloned DNA essentially as described by Klein *et al.* (1987), and introduced into *N. benthamiana* and *A. conyzoides* seedlings using either an Accell electric discharge gun as described by Cristou *et al.* (1990) or a portable helium flow particle gun using gas pressures varying from 100–500 psi. *A. conyzoides* plants ranging in size from newly emerging seedlings to plants at the four-leaf stage were selected for inoculation.

Analysis of viral DNA forms in infected tissues

Total cellular nucleic acids extracted from infected plants using the procedure of Covey and Hull (1981) were analyzed by Southern blotting after fractionation of samples in agarose gels containing TNE buffer. To identify ssDNA, 5-μg samples were treated with 5 u mungbean nuclease (BRL) in 300 mM sodium acetate, 500 mM NaCl, 10 mM $ZnCl_2$, 0.01% Triton X-100 (pH 4.6) for 1 h at 37°C. Blots were hybridized with probes specific for either AYVV coding sequences (*Bam*HI(136)–*Nco*I(1966) fragment; numbering according to Tan *et al.,* 1995), the intergenic region (described above) or defective DNA (using a fragment PCR-amplified from pAYVdef17 with primers V3445 and V3517; refer to Fig. 3). Viral DNA levels were estimated by scanning autoradiographs using a Bio-Rad GS-690 Imaging Densitometer.

Analysis of A. conyzoides genomic DNA

Genomic DNA was extracted from uninfected *A. conyzoides* plants using the method of Dellaporta *et al.* (1983). Samples (10 μg) were analyzed by Southern blotting after digestion with *Dra*I, *Eco*RV, or *Hin*dIII and agarose gel fractionation. Blots were hybridized with probes for defective DNA sequences from clones pAYVdef17 (described above) and pAYVdef19 (using a fragment PCR-amplified with primers corresponding to nucleotides 439–458 and 1119–1142 (complementary sense)). As a positive control, a host DNA probe was produced by PCR-amplification of a fragment of the *A. conyzoides* gene encoding 1-aminocyclopropane-1-carboxylic acid oxidase using degenerate primers (kindly provided by Dr. A. G. Prescott).

RESULTS

Blot hybridization using a probe specific for AYVV coding sequences (genomic DNA probe; Fig. 1A) showed that genome-sized ssDNA was the predominant viral DNA form associated with AYVV infection, either in *N. benthamiana* infected using cloned DNA (lane 1) or naturally infected *A. conyzoides* exhibiting yellow vein symptoms (lanes 3 and 4). The amount of scDNA was relatively low in comparison, reflecting the difficulty of isolating this viral DNA form for analytical purposes. However, sufficient scDNA was purified from *A. conyzoides* infected with a field isolate of AYVV to allow its detection using the genomic DNA probe (lane 5). The majority of the scDNA was linearized with *Bam*HI (lane 6) and produced *Eco*RI fragments of 1640 and 924 bp (lane 7), predicted from the nucleotide sequence (two other *Eco*RI fragments of 140 and 37 bp were too small to be detected in this assay). The presence of a minor amount of undigested scDNA (lane 6) and an *Eco*RI fragment migrating slightly faster than the linearized DNA (lane 7) is attributed to either genomic DNA sequence heterogeneity or copurifying contaminants from the plant extract that prevented complete digestion of the DNA.

In an attempt to identify a second genomic component analogous to DNA B of the bipartite viruses, the scDNA was analyzed using a probe specific for the intergenic region (Fig. 1B). Such a probe should detect putative common region sequences present on both genomic components. In untreated samples, the probe detected viral genomic scDNA as well as a faster migrating DNA that was not so evident using the genomic DNA probe (lane 5). Unlike the genomic DNA, the faster migrating DNA was not digested with *Bam*HI (lane 6) but was partially linearized with *Eco*RI (lane 7). All other DNAs that were detected with the intergenic region probe were also detected with the genomic DNA probe, implying that if a second component of comparable size to the characterized genomic DNA is associated with AYVV infection, it occurs at an extremely low level relative to the genomic DNA. A similar conclusion was reached following analysis of the scDNA using *Bgl*II and *Xho*II that have two and three sites, respectively, in the genomic DNA (data not shown).

The genomic DNA is highly infectious when agroinoculated into *N. benthamiana,* although repeated attempts to infect *A. conyzoides* by this method have been unsuccessful (Table 1; Tan *et al.,* 1995). In an attempt to define the infectious agent in *A. conyzoides,* the cloned genomic DNA was reintroduced into seedlings of this host using a biolistic delivery method. None of the plants developed symptoms after this treatment although almost 40% of *N. benthamiana* seedlings treated in the same manner developed symptoms typical of AYVV infection (Table 1). Similarly, no *A. conyzoides* plants developed symptoms following biolistic delivery of the scDNA although 40% of inoculated *N. benthamiana* plants became infected. No viral DNA was detected in upper asymptomatic *A. conyzoides* leaves by Southern blot analysis, although, a very low level of genome length double-stranded DNA was detected in the inoculated leaves of two plants inoculated with the cloned genomic DNA sampled 16 and 31 days postinoculation (data not shown), suggesting that the vi-

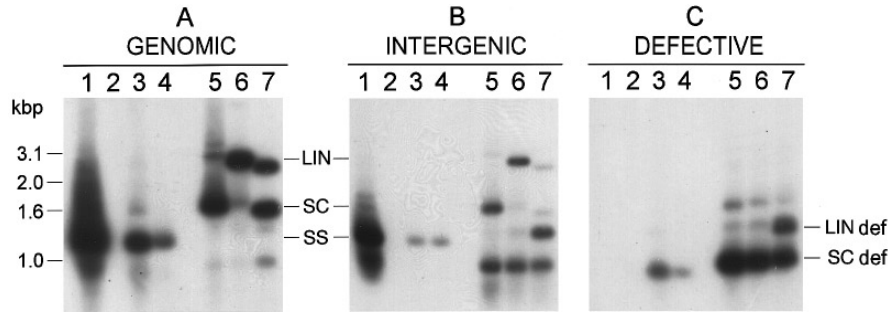

FIG. 1. Southern blot analysis of viral DNA forms associated with AYVV-infected plants. Total nucleic acids were isolated from an *N. benthamiana* plant infected using cloned genomic DNA (lane 1; 0.1 μg sample), from a healthy *A. conyzoides* plant (lane 2; 10 μg sample) and from two symptomatic *A. conyzoides* plants sampled from the same plot at the National University of Singapore in August 1995 and March 1996 (lanes 3 and 4, respectively; 10 μg samples). Samples (10 μl) of scDNA purified from infected *A. conyzoides* were either untreated (lane 5) or digested with *Bam*HI (lane 6) and *Eco*RI (lane 7). Blots were hybridized with probes specific for either viral coding sequences (A; Genomic), the intergenic region (B; Intergenic), or defective DNA (C; Defective), produced as described under Methods. The positions of genomic single-stranded (SS), supercoiled (SC), and linear (LIN) DNA forms, their defective counterparts (SCdef and LINdef), and size markers (kbp) are indicated.

ral DNA produced from the clone is at least able to replicate to some extent in this host.

To characterize the defective DNA, scDNA was digested with *Eco*RI and cloned into pIC19H. Virus-specific clones were identified by colony hybridization using a probe that will detect intergenic region sequences irrespective of their origin either from the genomic DNA or a putative DNA B component. The majority of virus-specific clones analyzed contained the 1640- and 924-bp *Eco*RI fragments originating from the genomic DNA, although six clones contained inserts approximating in size to that expected of the defective DNA. Sequence analysis revealed that all of these clones contained most if not all of the intergenic region in addition to variable amounts of the 5′ terminal coding regions of genes *C1, C4,* and *V2* (Fig. 2). The remaining sequences showed no similarity to any other geminivirus sequence (Fig. 3) and are of unknown origin (referred to here as nonviral sequences). The fact that sequences on either side of the *Eco*RI cloning site are contiguous within the AYVV intergenic region implies that all of the clones derive from circular defective DNAs, each approximately half the size (pAYVdef17, 1305 bp; pAYVdef19, 1343 bp; pAYVdef27, 1336 bp; pAYVdef41, 1324 bp) of the genomic DNA (2741 bp; Tan *et al.,* 1995). The sequences of pAYVdef20 and pAYVdef42 inserts were identical to those of pAYVdef41 and pAYVdef17, respectively. The defective DNAs in clones pAYVdef17, pAYVdef27, and pAYVdef41 clearly have a common origin, but contain different deletions within both viral and nonviral sequences (Fig. 3). In contrast, the nonviral sequences of clone pAYVdef19 were dissimilar to those associated with the other clones and contained additional genomic DNA sequences from the coat protein coding region, corresponding to nucleotides 497–746, embedded within them (Fig. 2), implying that this defective DNA was produced by at least two distinct recombination events.

Using a probe specific for the nonviral sequences of pAYVdef17 we were able to demonstrate that defective DNA containing similar nonviral sequences was maintained in individual *A. conyzoides* plants sampled from Singapore over a 7-month period (Fig. 1C, lanes 3 and 4). The same probe also readily detected the defective scDNA isolated from laboratory-maintained plants that

TABLE 1

Systemic Infectivity of AYVV in *N. benthamiana* and *A. conyzoides*

Delivery method	Inoculum	*N. benthamiana*[a]	*A. conyzoides*[a]
Biolistic[b]	scDNA	4/10 (1)	0/65 (3)
	Genomic DNA	6/16 (2)	0/83 (4)
Agroinoculation[c]	Genomic DNA	28/35 (4)	0/5 (1)
	Genomic DNA + defective DNA	26/26 (3)	0/10 (1)
	Genomic DNA + defective DNA mutant	10/10 (1)	Not done

[a] Number of plants systemically infected/number of plants inoculated (number of experiments). Infection was determined by the development of symptoms. Dot blot analysis was used to ensure that asymptomatic tissues did not contain detectable levels of viral DNA.

[b] Plants were challenged with either scDNA isolated from naturally infected *A. conyzoides* plants or a cloned partial repeat of the AYVV genomic DNA (clone pHNIC419; Tan *et al.,* 1995).

[c] Plants were agroinoculated with AYVV genomic DNA (clone pHNBin419; Tan *et al.,* 1995) alone or in the presence of either the defective DNA (clone pBinAYVdef17) or its mutant derivative (clone pBinAYVdef17ΔC1).

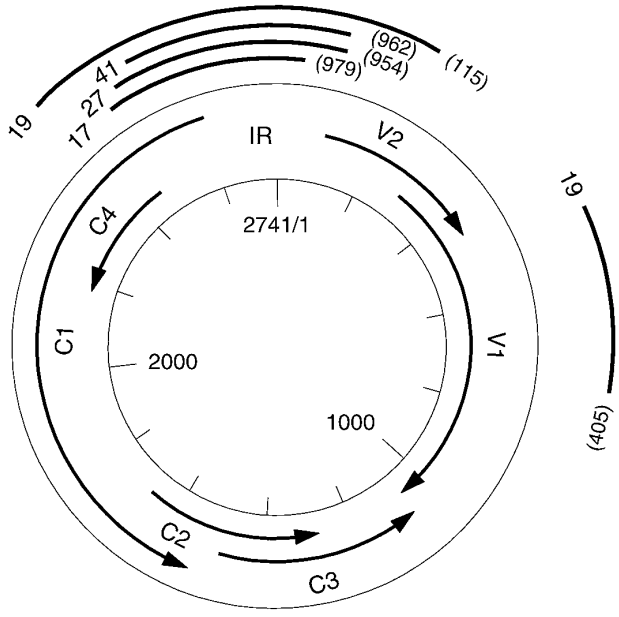

FIG. 2. AYVV DNA sequences retained within the defective viral DNAs. The location of virion-sense genes (V1 and V2), complementary-sense genes (C1–C4), and intergenic region (IR) are indicated within the genomic map of AYVV. AYVV sequences retained within clones pAYVdef17, pAYVdef19, pAYVdef27, and pAYVdef41 are indicated on the outside of the map. Figures in parentheses indicate the number of nonviral nucleotides associated with each defective DNA.

had been infected by whitefly transmission from one of the original field isolates (lanes 5 to 7). In contrast, the nonviral sequences were not detected in agroinoculated *N. benthamiana* plants (lane 1).

Database searches using both the nonviral DNA sequences and the putative translation products of their small open reading frames failed to reveal homologies that might suggest a possible origin for the nonviral DNA. To investigate if the nonviral DNA originated from the host plant, *A. conyzoides* genomic DNA was isolated, digested with restriction enzymes, and probed for nonviral sequences from pAYVdef17 and pAYVdef19 by blot hybridization. Neither probe detected fragments of the host genomic DNA, although fragments were readily detected when control blots were probed for host gene sequences using a fragment of the gene encoding 1-aminocyclopropane-1-carboxylic acid oxidase (data not shown).

To investigate a possible biological role for the AYVV defective DNA, one example (derived from clone pAYVdef17) was reintroduced into plants by agroinoculation in the presence of AYVV genomic DNA. *A. conyzoides* plants coagroinoculated with the genomic and defective DNA did not develop symptoms, and no viral DNA was detected in the upper asymptomatic leaves by Southern blot analysis (Table 1). Approximately 2 weeks after inoculation, the majority of *N. benthamiana* plants inoculated with the genomic DNA alone became infected, all of which developed downward leaf curl symptoms that rapidly developed into a severe upward leaf roll symptom typical of AYVV infection (Table 1; Fig. 4A). In experiments conducted at the same time, all plants coinoculated with the genomic and defective DNAs also became infected, but typically developed the downward leaf curl symptom (Fig. 4A) and only sporadically developed very mild upward leaf roll. These distinct phenotypes were retained over the duration of each experiment (up to 2 months postinoculation). All infected plants developed tissue swelling symptoms typical of AYVV infection of *N. benthamiana,* irrespective of the presence or absence of the defective DNA. For example, developmental abnormalities associated with the flowers of infected plants are clearly visible in Fig. 4B.

Southern blot analysis of viral DNA extracted from individual leaves harvested 19 days postinoculation indicated that the defective DNA was maintained during systemic infection (Fig. 5A). Image densitometry of the autoradiograph showed that the accumulation of the genomic DNA was reduced on average by 78% in the presence of the defective DNA (compare lanes 1 and 2 with lanes 3–7). On average the defective DNA accumulated to 23% of the total viral DNA in the systemically infected tissues. However, 2 months postinoculation, the level of genomic DNA in plants coagroinoculated with the defective DNA (Fig. 5B, lanes 4–6) was reduced on average by only 13% compared with plants agroinoculated with genomic DNA alone (lanes 1–3). Also, the relative level of defective DNA increased at this later stage of infection and slightly exceeded that of the genomic DNA.

The defective DNA used in these experiments retained the capacity to encode 43 N-terminal amino acids of the C1 protein fused to an additional 37 amino acids encoded by the adjacent nonviral DNA. Since overexpression of an N-terminal fragment of AC1 protein has been shown to have an adverse effect on ACMV DNA accumulation in *N. tabacum* protoplasts (Hong and Stanley, 1996) and on TYLCV DNA accumulation in transgenic *N. benthamiana* plants (Noris *et al.,* 1996), we wished to know if expression of the chimeric C1 protein from the AYVV defective DNA contributed to the interference phenomenon. Accordingly, expression of the C1 protein was disrupted by introduction of a frameshift mutation after the fifth amino acid of the *C1* coding region. All plants coinfected with this mutant and the genomic DNA developed the downward leaf curl symptoms similar to those associated with the unmodified defective DNA (Table 1; Fig. 4), and the accumulation of viral DNA forms in plants containing the modified and unmodified defective DNAs was indistinguishable (Fig. 5B, compare lanes 4–6 with lanes 7–12), indicating that the chimeric C1 protein does not contribute significantly to the phenotype.

Defective DNA forms were synthesized *de novo* in *N. benthamiana* plants infected with the genomic DNA alone. They were barely detectable 19 days postinoculation (only when the autoradiograph of Fig. 5A was overex-

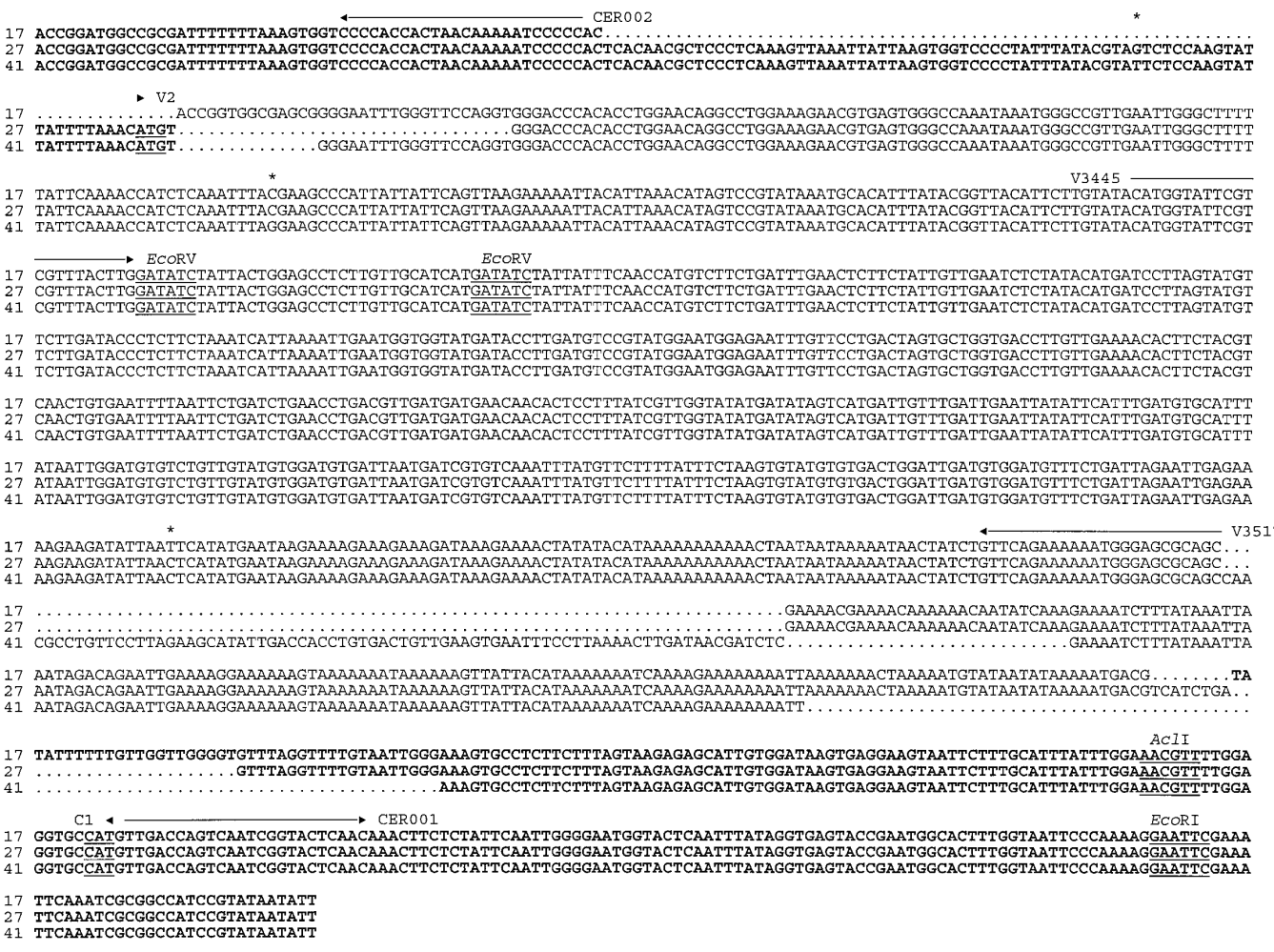

FIG. 3. Nucleotide sequences of the inserts of AYVV defective DNA clones. The sequences correspond to the virion-sense strand of defective DNA clones pAYVdef17, pAYVdef27, and pAYVdef41, and position 1 has been defined as the A residue immediately downstream of the nick site within the conserved nonanucleotide (TAATATT↓AC) (Laufs *et al.,* 1995; Stanley, 1995). Nucleotides that occur in AYVV genomic DNA are in boldface. Asterisks indicate positions of sequence heterogeneity. The nucleotide triplets corresponding to the initiation codons of virion-sense gene *V2* and complementary-sense gene *C1* (Tan *et al.,* 1995), and the *Eco*RI, *Eco*RV, and *Acl*I sites used for cloning a partial repeat of the pAYVdef17 insert and mutagenesis are underlined. The positions and orientations of primers CER001, CER002, V3445, and V3517 used to produce hybridization probes are indicated.

posed) but accumulated over a 2-month period (Fig. 5B, lanes 1–3). The newly synthesized defective DNAs were significantly smaller than those isolated from *A. conyzoides,* and it is interesting to note that similar viral DNA forms did not accumulate when plants were coagroinoculated with the cloned defective DNA (Fig. 5B, compare lanes 1–3 with lanes 4–12).

DISCUSSION

We have previously shown that a single genomic component of AYVV is infectious in *N. benthamiana,* French bean, and tomato but is unable to reinfect the original host *A. conyzoides* by either agroinoculation of the infectious cloned DNA or whitefly transmission of the cloned DNA progeny (Tan *et al.,* 1995). Furthermore, although yellow vein disease was whitefly transmissible between *A. conyzoides* plants, and AYVV was transmissible in this manner to French bean and tomato, the virus originating from either the wild-type population or cloned DNA was not whitefly transmissible to any host using infected French bean and tomato as a virus source (Tan *et al.,* 1995). This prompted the suggestion that an additional factor, such as a second genomic component analogous to DNA B, may be associated with yellow vein disease of *A. conyzoides* but was not required for infection of more permissive hosts. In an attempt to identify other viral DNAs that may contribute to infection of *A. conyzoides,* viral scDNA was purified from naturally infected plants and analyzed. Two lines of evidence suggest that a second genomic DNA component does not occur in these plants. First, blot hybridization studies on the scDNA using a probe designed to detect putative common region sequences revealed only fragments from the

FIG. 4. Symptom amelioration in *N. benthamiana* caused by AYVV defective DNA. (A) Symptomatic leaves of plants infected with genomic DNA, either alone (bottom left) or in the presence of defective DNA (bottom right), photographed 19 days after inoculation. Equivalent leaves from a mock-inoculated plant (top) are shown for comparison. (B) Flowers from a healthy plant (left) and plants infected with genomic DNA, either alone (center) or in the presence of defective DNA (right). Flowers from both infected plants have developed tissue abnormalities typical of AYVV infection of this host.

genomic or defective DNA, indicating that if a second genomic component exists, it occurs at an extremely low level. This would contrast with reports on bipartite geminiviruses such as ACMV (Stanley, 1983) and TGMV (Hamilton *et al.,* 1983), which indicate that both genomic DNAs occur at similar levels. Furthermore, studies on the viability and pathogenicity of pseudorecombinants have suggested that a strong selection pressure exists for the maintenance of similar levels of both genomic components (Hou and Gilbertson, 1996). Second, attempts to infect *A. conyzoides* by biolistic delivery of scDNA isolated from this host, which would be expected to contain functional amounts of all viral DNA components, were unsuccessful. We cannot yet rule out the possibility that the biolistic method is ineffective in delivering the inoculum to the appropriate tissues of this host, although the scDNA initiated infections in *N. benthamiana* in control experiments. Alternatively, the purified scDNA itself may lack an essential factor necessary for infection in *A. conyzoides.*

The search for an additional component revealed a population of defective DNAs associated with yellow vein disease of *A. conyzoides.* All of these defective DNAs clearly derive from the genomic DNA because they con-

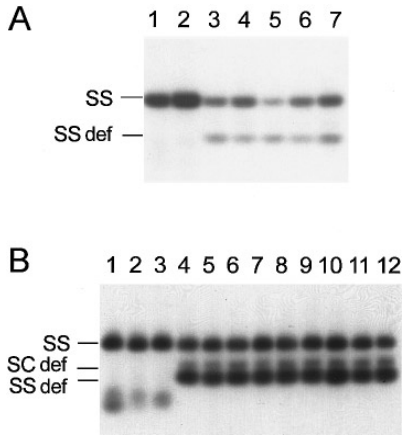

FIG. 5. Southern blot analysis of AYVV genomic and defective DNA forms produced in agroinoculated *N. benthamiana*. (A) Total nucleic acids were isolated from single leaves from individual plants inoculated with genomic DNA, either alone (lanes 1 and 2) or in the presence of defective DNA (lanes 3 to 7). Leaves were sampled 19 days after inoculation. Sampled leaves exhibited symptoms of upward rolling (lanes 1 and 2), downward curling (lanes 3 to 6), and downward curling with very mild upward rolling at the edge (lane 7). (B) Total nucleic acids were isolated from comparable groups of upper leaves from individual plants inoculated with genomic DNA, either alone (lanes 1 to 3) or in the presence of defective DNA (lanes 4 to 6) or the defective DNA mutant containing a frameshift mutation within gene *C1* (lanes 7 to 12). Leaves were sampled 2 months after inoculation. Blots were hybridized with a probe specific for the intergenic region of the genomic DNA. The positions of genomic single-stranded DNA (SS) and its defective DNA counterpart (SSdef), identified by their susceptibility to mungbean nuclease digestion (data not shown), and supercoiled defective DNA (SCdef) are indicated.

tain sequences from the gene *C1* coding region, and they all retain at least part of the intergenic region that contains *cis*-acting elements required for viral DNA replication (reviewed by Bisaro (1996)). However, they are unique in the respect that the majority of their sequence appears to be nonviral in origin. It was anticipated that these nonviral sequences originated from the host plant, *A. conyzoides,* although we have been unable to confirm this by genomic blot hybridization. It is possible that rearrangments have occurred in these sequences over time to the extent that they no longer cross-hybridize with host DNA. Alternatively, the nonviral sequences may have originated from an entirely different host species.

The defective DNA population was maintained during insect transmission of the virus between *A. conyzoides* plants, implying that the defective DNA spreads systemically throughout the plant and suggesting that it is encapsidated in order to be whitefly transmitted. A combination of factors such as a stringent size requirement for systemic movement of the viral DNA (Etessami *et al.,* 1989; Klinkenberg *et al.,* 1989) and packaging constraints within icosahedral or twinned quasi-icosahedral (geminate) particles may select for defective DNA of approximately half the size of the genomic DNA.

Defective DNAs associated with ACMV and TGMV infection (Stanley and Townsend, 1985; MacDowell *et al.,* 1986) are of a similar size to the AYVV defective DNAs and are also maintained during plant-to-plant transmission (Stanley *et al.,* 1990). Unlike AYVV defective DNA, those associated with ACMV and TGMV infection all derive from the DNA B component by deletion of coding sequences. Defective DNAs also occur in plants infected with beet curly top virus (BCTV) (Frischmuth and Stanley, 1992; Stenger *et al.,* 1992). The BCTV defective DNAs are produced simply by deletion of sequences from the genomic component (equivalent to AYVV genomic DNA) and contain no nonviral sequences. They occur in a much wider size range (800–1800 bp) and are rapidly produced *de novo* during the infection of *N. benthamiana.* Defective DNAs are also produced *de novo* in *N. benthamiana* plants infected with AYVV genomic DNA alone, although they are significantly smaller than those originating from *A. conyzoides* and may represent precursors of the larger defective DNAs that eventually acquire nonviral sequences. Interestingly, these very small DNAs remained undetected when the genomic DNA was coinoculated with the cloned defective DNA. Similarly, a single defective DNA produced from an integrated tandem repeat was the predominant form in BCTV-infected transgenic *N. benthamiana* plants, even though a range of different sized DNAs accumulated in nontransgenic control plants (Frischmuth and Stanley, 1994). This suggests that newly generated defective DNAs are not retained in the population due to competition from the existing defective DNA that is particularly well adapted for replication and/or systemic movement.

A circular satellite DNA has recently been isolated from TLCV-infected tomato (Dry *et al.,* 1997). The satellite DNA differs from the AYVV defective DNA in that it is approximately half the size (682 nucleotides) and shows no obvious sequence homology with its helper virus apart from some *cis*-acting elements involved in viral DNA replication including the conserved nonanucleotide TAATATTAC located within a potential stem–loop structure that is a feature of all geminiviruses. Hence, it is not clear if the TLCV satellite DNA originated from the helper virus, as suggested for the AYVV defective DNA, or if it is a product of convergent evolution. In many respects, the AYVV defective DNA resembles turnip crinkle virus RNA C, which contains sequences of the helper virus in addition to unrelated sequences (Simon and Howell, 1986).

Inspection of the defective DNA sequences suggests possible mechanisms by which the defective DNA acquires the nonviral sequences. First, the 5′-terminal sequence of pAYVdef17 nonviral DNA, ACCGG (Fig. 3), is identical to the AYVV sequence immediately downstream of the nick site within the origin of virion-sense DNA replication (Laufs *et al.,* 1995) and may contribute to a cryptic origin. It is known that the nick site within the origin of replication is a recombinational hot spot (Etes-

sami *et al.,* 1989; Stanley, 1995) that may play an important role in geminivirus evolution (Hou and Gilbertson, 1996). Second, the 5′-terminal sequence of pAYVdef27 and pAYVdef41 nonviral DNA, GGGA in both cases (Fig. 3), is also present in AYVV genomic DNA immediately downstream of the point of recombination, suggesting that it participates in locating the crossover point in these particular defective DNAs. Previous studies have indicated that such small repeat sequences may define the deletion end-points during the production of ACMV, TGMV, and BCTV defective DNAs (Stanley and Townsend, 1985; MacDowell *et al.,* 1986; Frischmuth and Stanley, 1992).

The defective DNA altered the symptom phenotype and caused a delay in the accumulation of viral DNA when reintroduced into *N. benthamiana* in the presence of AYVV genomic DNA. The severe upward leaf roll symptom that is characteristic of infection of *N. benthamiana* by single component geminiviruses such as AYVV and BCTV (Stanley and Latham, 1992; Tan *et al.,* 1995) developed only rarely and in a very mild form in the presence of the defective DNA. The mild downward leaf curl phenotype associated with the presence of the defective DNA is reminiscent of that induced by BCTV *C4* mutants (Stanley and Latham, 1992), except that the vein swelling symptoms and other morphological abnormalities normally associated with these viruses, but absent in plants infected with *C4* mutants, still occur in the presence of the defective DNA. The mild phenotype was retained even at a late stage of infection when the level of AYVV genomic DNA approached that in plants exhibiting the severe phenotype. This suggests that the leaf curl phenotype is determined by the ability of the virus to affect certain tissues at an early stage of their development rather than by the overall level of virus that accumulates in these tissues. The behavior of the AYVV defective DNA resembles that of ACMV and BCTV defective DNAs that have been shown to function as defective interfering (DI) DNAs (Stanley and Townsend, 1985; Stanley *et al.,* 1990; Frischmuth and Stanley, 1994; Stenger, 1994). Therefore, the AYVV defective DNAs may perform a biological role by slowing down the infection process that may otherwise be deleterious to the plant and, hence, disadvantageous for the maintenance of the virus population.

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